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Noncovalent Attachment of NAD^+ Cofactor onto Carbon Nanotubes for Preparation of Integrated Dehydrogenase-Based Electrochemical Biosensors

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Received October 3, 2009. Revised Manuscript Received January 13, 2010

This study describes a facile approach to the preparation of integrated dehydrogenase-based electrochemical biosensors through noncovalent attachment of an oxidized form of β-nicotinamide adenine dinucleotide (NAD^+) onto carbon nanotubes with the interaction between the adenine subunit in NAD^+ molecules and multiwalled carbon nanotubes (MWCNTs). X-ray photoelectron spectroscopic and cyclic voltammetric results suggest that NAD^+ is noncovalently attached onto MWCNTs to form an NAD^+/MWCNT composite that acts as the electronic transducer for the integrated dehydrogenase-based electrochemical biosensors. With glucose dehydrogenase (GDH) as a model dehydrogenase-based recognition unit, electrochemical studies reveal that glucose is readily oxidized at the GDH/NAD^+/MWCNT-modified electrode without addition of NAD^+ in the phosphate buffer. The potential for the oxidation of glucose at the GDH/NAD^+/MWCNT-modified electrode remains very close to that for NADH oxidation at the MWCNT-modified electrode, but it is more negative than those for the oxidation of glucose at the MWCNT-modified electrode and for NADH oxidation at a bare glassy carbon electrode. These results demonstrate that NAD^+ molecules stably attached onto MWCNTs efficiently act as the cofactor for the dehydrogenases. MWCNTs employed here not only serve as the electronic transducer and the support to confine NAD^+ cofactor onto the electrode surface, but also act as the electrocatalyst for NADH oxidation in the dehydrogenase-based electrochemical biosensors. At the GDH/NAD^+/MWCNT-based glucose biosensor, the current is linear with the concentration of glucose being within a concentration range from 10 to 300 μM with a limit of detection down to 4.81 μM ($S/N = 3$). This study offers a facile and versatile approach to the development of integrated dehydrogenase-based electrochemical devices, such as electrochemical biosensors and biofuel cells.

Introduction

Considerable interests have been drawn in the development of electrochemical biosensors because the combination of the advantages of electrochemical methods with the specific recognition properties of biorecognition units (e.g., enzymes and proteins) has substantially endowed electrochemical biosensors with excellent properties and attractive applications.[1] To this end, the last several decades have witnessed great progress not only in the fundamental studies but also in the practical applications of electrochemical biosensors.[2] While much effort has been made in establishing bioelectrocatalytic schemes involved in electrochemical biosensing and in confining enzyme biorecognition units onto electronic transducers,[3] the pressing need for fast and cost-effective as well as environmentally benign measurements substantially necessitates an effective integration of all components involved in the bioelectrocatalytic reactions onto the electronic transducers. This is because dissolving of either electron-transfer mediators or enzyme cofactors into solution phase, on one hand, makes the determinations time-consuming and expensive and, on the other hand, results in an environmental burden. Nevertheless, the good solubility in the aqueous solutions of the commonly used electron-transfer mediators, such as ferricyanide generally used to shuttle the electron transfer of flavin adenine dinucleotide (FAD)-based oxidases, and β-nicotinamide adenine dinucleotide (NAD^+) cofactor for more than 300 kinds of dehydrogenases substantially renders a long-standing challenge for the development of integrated electrochemical biosensors.[4]

In our earlier attempt, we have demonstrated a method to effectively confine ferricyanide redox mediator onto electronic transducers through rationally functionalizing carbon nanotubes with positively charged methylimidazolium moieties, which could pave a general route to the development of integrated oxidase-based electrochemical biosensors.[5] In this study, we present a

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simple method to confine NAD^+ cofactor onto electronic transducers for generally developing integrated dehydrogenase-based electrochemical biosensors. Previous excellent strategies employed for surface confinement of NAD^+ cofactor in the dehydrogenase-based electrochemical biosensors have included comixing NAD^+ cofactor with dehydrogenases into carbon paste electrodes[6] and covalently anchoring NAD^+ cofactor onto the electronic transducers by introducing aminoethyl or carboxymethyl groups into the adenine subunit in NAD^+[7] or through the interaction between phenylboronic acid units on the electronic transducer and pentose moiety of NAD^+.[8] The successful confinement of NAD^+ cofactor onto the electronic transducers has facilitated the development of integrated electrochemical biosensors and represented a great advance in this research filed.

The rationale for the strategy demonstrated here for simple confinement of NAD^+ cofactor onto the electronic transducers is basically based on the strong $\pi-\pi$ stacking interaction between the adenine subunit in the NAD^+ molecule and carbon nanotubes (CNTs).[9] As a new member in the carbon family, CNTs have unique structural and electronic properties and thus offer striking applications in various research and industrial fields.[10] Research in recent years has revealed that CNTs are rich in surface chemistry, which further broadens their applications through covalent and noncovalent functionalization protocols.[11] In addition to their special surface chemistry that essentially facilitates the surface confinement of the NAD^+ molecule, the good conductivity and the distinct structural properties of CNTs substantially enable them to act as electronic transducers and as electrocatalysts for NADH oxidation in integrated dehydrogenase-based biosensors. Compared with the methods previously employed for surface confinement of NAD^+, the strategy demonstrated here avoids the complex synthetic procedures and thereby offers a simple but effective approach to the general development of integrated dehydrogenase-based biosensors and biofuel cells.

Experimental Section

Chemicals and Materials. Both kinds of multiwalled carbon nanotubes (MWCNTs) with diameters of less than 30 and 10 nm and single-walled carbon nanotubes (SWCNTs) with diameters of less than 2 nm were all purchased from Shenzhen Nanoport Co. Ltd. (Shenzhen, China). The nanotubes were purified by refluxing the as-received samples in 2.6 M HNO_3 for 10 h. Glucose dehydrogenase (GDH, EC 1.1.3.4, from *Aspergillus niger*) and D-(+)-glucose were obtained from Sigma and used as supplied. Reduced and oxidized forms of nicotinamide adenine dinucleotide (NADH and NAD^+) were purchased from Aldrich. Bovine serum albumin (BSA), glutaraldehyde (1% aqueous solution), and glucose were all purchased from Beijing Chemical Reagent Co. Ltd. (Beijing, China). Other chemicals were of analytical grade or higher and used as received. Aqueous solutions were prepared with doubly distilled water.

Preparation of NAD^+/MWCNT Composite. The NAD^+/MWCNT composite was prepared by mixing 2 mg of MWCNTs and 40 mg of NAD^+ in 4 mL of distilled water under stirring for 48 h at room temperature. The resulting suspensions were filtered with a Millipore filter (0.45 μm, Millipore), and the obtained samples were first thoroughly rinsed with distilled water to remove nonadsorbed NAD^+ onto MWCNTs and then dried at 40 °C under vacuum overnight to obtain the NAD^+/MWCNT composite.

Preparation of Glucose Biosensor. Glassy carbon electrodes (GC, 3 mm diameter, Bioanalytical Systems, Inc.) were used as the substrate for the development of electrochemical biosensors with the NAD^+/MWCNT composite as the electronic transducer. Pt electrodes (1.6 mm diameter, Bioanalytical Systems, Inc.) were used as the substrate to verify the adsorption of NAD^+ cofactor onto MWCNTs. The electrodes were polished first with emery paper and then with aqueous slurries of fine alumina powders (0.3 and 0.05 μm) on a polishing cloth. The electrodes were then rinsed with ethanol and doubly distilled water in an ultrasonic bath, each for 2 min, and finally with distilled water. The synthetic NAD^+/MWCNT composite was dispersed into ethanol with sonication to obtain a homogeneous dispersion (2 mg/mL), and 2 μL of the resulting dispersion was dip-coated onto GC electrodes. The electrodes (denoted as NAD^+/MWCNT-modified GC) were then dried at ambient temperature. Alternatively, the NAD^+/MWCNT-modified GC electrodes could also be prepared by first dip-coating 4 μL of MWCNT dispersion in dimethylformamide (DMF, 2 mg/mL) onto GC electrodes and then immersing the MWCNT-modified GC electrodes in the aqueous solution of 10 mM NAD^+ for about 12 h for saturated adsorption of NAD^+ cofactor onto the MWCNTs. The electrodes were finally rinsed with doubly distilled water and dried at ambient temperature. GDH was used as the model biorecognition unit to demonstrate the generality of the development of the electrochemical biosensors with NAD^+/MWCNT as the electronic transducer. For this purpose, 2 μL of an aqueous solution of GDH (10 mg/mL) was mixed with 1 μL of an aqueous solution of BSA (1%) to give a GDH−BSA mixture that was totally dip-coated onto the NAD^+/MWCNT-modified GC electrodes. After that, 1 μL of glutaraldehyde aqueous solution (1%) was pipetted onto the electrode surface to cross-link GDH coated onto MWCNTs through the interaction between glutaraldehyde and the amine groups in GDH. The resulting electrodes (denoted as GDH/NAD^+/MWCNT-modified GC) were dried at ambient temperature and stored at +4 °C in a refrigerator while not in use. Cyclic voltammetric measurements conducted with the GDH/NAD^+/MWCNT-modified GC electrodes were subjected to automatic compensation of the ohmic potential drop.

Apparatus and Measurements. Electrochemical measurements were carried out on a computer-controlled potentiostat (Autolab, PGSTAT 302, Metrohm, Switzerland) in a two-compartment electrochemical cell with the modified GC and Pt electrodes as working electrode, a platinum spiral wire as counter electrode, and a Ag/AgCl electrode (saturated with KCl) as reference electrode. Pt electrodes were used as the substrate electrode for voltammetric characterization of NAD^+ adsorption onto MWCNTs because, unlike carbon electrodes, this kind of electrode was not electrochemically activated to produce quinone-like groups that are redox-active at ca. 0.0 V. A 0.10 M phosphate buffer (pH 7.0) was used as supporting electrolyte. X-ray photoelectron spectroscopy (XPS) was performed on an ESCALab-220i-XL electron spectrometer from VG Scientific using 300 W Al KR radiation.

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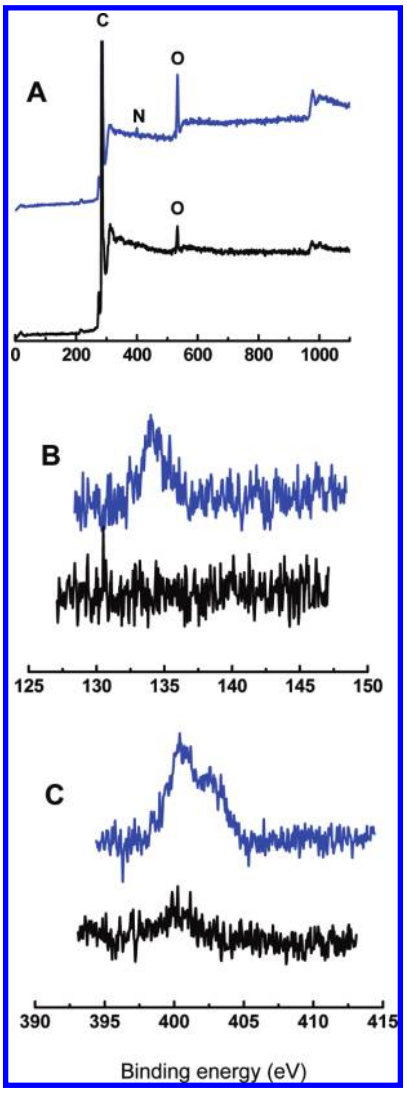

Figure 1. XPS spectra (A) and high-resolution XPS spectra of P2p (B) and N1s (C) of pristine MWCNTs (black curves) and NAD^+/MWCNT composite (blue curves).

Results and Discussion

NAD^+ Adsorption onto MWCNTs. Figure 1 displays the XPS spectra of pristine MWCNTs (black curve) and the NAD^+/MWCNT composite (blue curve). As shown in panel A, the peaks at 284.8 and 533.8 eV in the spectra of the NAD^+/MWCNT composite (blue curve) and pristine MWCNTs (black curve) were, respectively, ascribed to the carbon atoms (C1s) and oxygen atoms (O1s) of MWCNTs and/or NAD^+.[12] The appearance of the peaks at 134.0 eV (panel B) and 400.5 eV (panel C) in the spectra of the NAD^+/MWCNT composites, which were ascribed to the phosphor atoms (P2p) and nitrogen atoms (N1s), suggests the adsorption of NAD^+ cofactor onto MWCNTs.[12]

The adsorption of NAD^+ cofactor onto MWCNTs was also verified from cyclic voltammograms (CVs) obtained with MWCNT-modified Pt electrodes after the electrodes were first immersed into the aqueous solution of 10 mM NAD^+ for 1 h and then thoroughly rinsed with distilled water, as depicted in Figure 2. Instead of GC electrodes, Pt electrodes were used in this case because this kind of electrode was not electrochemically activated

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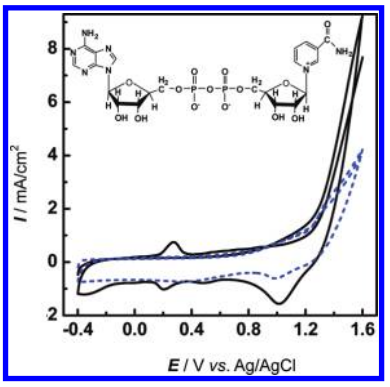

Figure 2. CVs obtained at the MWCNT-modified Pt electrodes in 0.10 phosphate buffer before (blue curve) and after (black curve) the electrodes were first immersed into the aqueous solution of 10 mM NAD^+ for 1 h and then thoroughly rinsed with distilled water. Initial potential, −0.40 V; scan rate, 50 mV s^{-1}. Inset: structure of NAD^+ cofactor.

at a high potential to produce surface quinone-like groups at ca. 0.0 V. After being immersed into the aqueous solution of 10 mM NAD^+, the MWCNT-modified Pt electrode exhibits a tailed oxidation process commencing at +1.40 V in the first anodic scan and a pair of well-defined redox waves at +0.20 V in the subsequent scans (black curve). These redox waves were not recorded at the MWCNT-modified Pt electrode before the electrode was immersed into the NAD^+ solution (blue curve). The tailed oxidation peak was ascribed to the oxidation of the adenine subunit in the NAD^+ molecules, while the redox wave at +0.20 V was attributed to the redox process of the oxidation product of NAD^+, that is, a quinone-diimine compound, as reported previously.[6c,9c] The appearance of these redox peaks at the MWCNT-modified Pt electrode substantially suggests the adsorption of NAD^+ molecules onto MWCNTs to form the NAD^+/MWCNT composite. The adsorption of NAD^+ molecules onto MWCNTs was quite stable, which might be indirectly evident from the unchanged redox peak currents at +0.20 V after consecutive potential cycling for at least 50 cycles in 0.10 M phosphate buffer (pH 7.0) (data not shown). The stable adsorption of NAD^+ onto MWCNTs was considered to result from the strong $\pi-\pi$ stacking interaction between the adenine subunit in the NAD^+ molecule and MWCNTs, as reported previously.[9]

The adsorption of NAD^+ onto MWCNTs to form the NAD^+/MWCNT composite was further confirmed by following two control experiments: one by changing the sort of CNTs and the other by changing the time for immersing the MWCNT-modified electrodes into the NAD^+ solution. In the former control experiments, we studied the NAD^+ adsorption onto three kinds of CNTs, that is, one kind of SWCNTs with a diameter less than 2 nm and two kinds of MWCNTs with diameters less than 10 and 30 nm. After being immersed into 10 mM NAD^+ solution for 1 h, all Pt electrodes modified with each kind of CNTs exhibited almost the same redox waves as those at the NAD^+/MWCNT-modified electrode shown in Figure 2 (black curve) in 0.10 M phosphate buffer (pH 7.0). In the latter control experiments, the MWCNT-modified Pt electrodes were immersed into 10 mM NAD^+ solution for different times. As displayed in Figure 3, the peak currents for the redox wave at +0.20 V increase with increasing immersion time, suggesting the adsorption of NAD^+ onto MWCNTs. These results substantially demonstrate that the strong $\pi-\pi$ stacking interaction between the adenine subunit in the NAD^+ molecule and MWCNTs eventually leads to the stable attachment of the NAD^+ cofactor onto MWCNTs. Such a

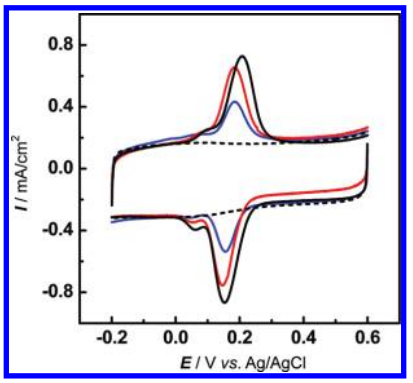

Figure 3. CVs obtained at the MWCNT-modified Pt electrodes in 0.10 M phosphate buffer (pH 7.0) after the electrodes were first immersed into the 10 mM NAD^+ aqueous solution for different times of 5 min (blue curve), 1 h (red curve), and 2 h (black curve) and then rinsed with distilled water. Short-dashed curve represents the CV of the MWCNT-modified Pt electrodes in 0.10 M phosphate buffer (pH 7.0) without immersing the electrode into the NAD^+ aqueous solution. Prior to CV measurements, the electrodes were scanned within a potential range from −0.20 to +1.60 V for three cycles to oxidize the NAD^+ cofactor adsorbed onto MWCNTs. Scan rate, 50 mV s^{-1}.

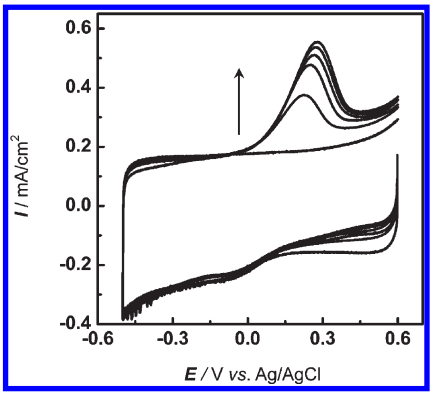

Figure 4. CVs for the GDH/NAD^+/MWCNT-modified GC electrode in 0.10 M phosphate buffer (pH 7.0) containing glucose with different concentrations of 0, 5, 10, 20, 40, and 80 mM. Scan rate, 50 mV s^{-1}.

property actually enables the development of integrated dehydrogenase-based electrochemical biosensors with the NAD^+/MWCNT composite as the electronic transducer, as described below.

Toward Electrochemical Biosensing. By using GDH as the model biorecognition element, we demonstrated the use of the NAD^+/MWCNT composite as an electronic transducer for the development of integrated electrochemical biosensors for glucose. Figure 4 depicts typical cyclic voltammograms of glucose oxidation at the GDH/NAD^+/MWCNT-modified GC electrode without the presence of NAD^+ cofactor in the phosphate buffer. The oxidation of glucose occurs at the electrode at +0.25 V with the peak currents clearly increasing with the concentration of glucose in solution. For comparison, we immersed a bare GC electrode (i.e., without MWCNT modification) into the 10 mM NAD^+ solution for 12 h and then cross-linked GDH onto the electrode with the same procedures as those used for the MWCNT-modified electrodes and finally studied the electrochemical response toward glucose in 0.10 M phosphate buffer containing no NAD^+. Different from the case of the GDH/NAD^+/MWCNT-modified electrodes, the electrode prepared without initial modification of the MWCNT layer does not show

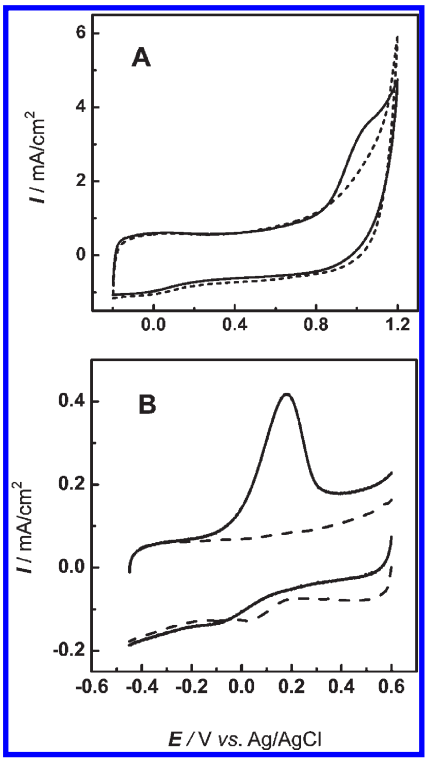

Figure 5. CVs for the MWCNT-modified GC electrodes in 0.10 M phosphate buffer (pH 7.0) in the absence (short dashed curves) and presence (solid curves) of 40 mM glucose (A) or 4 mM NADH (B). Scan rate, 50 mV s^{-1}.

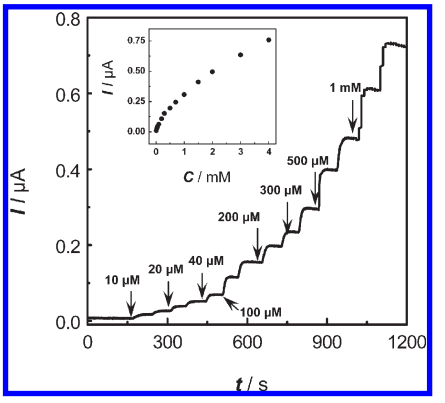

Figure 6. Typical amperometric response obtained at the GDH/NAD^+/MWCNT-modified GC electrodes in 0.10 M phosphate buffer solution (pH 7.0) toward successive addition of glucose with the concentrations indicated in the figure. Applied potential, +0.30 V. Inset: plot of current response versus concentration of glucose.

a faradic response toward glucose (data not shown), further indicating that the NAD^+ cofactor was adsorbed only onto MWCNTs. These results demonstrate the successful immobilization of NAD^+ cofactor onto the MWCNT electronic transducer essentially forms a straightforward basis for the development of integrated dehydrogenase-based biosensors.

The GDH-catalyzed oxidation of glucose with surface-immobilized NAD^+ as the cofactor and with the oxidation of the reduced NAD^+ cofactor (i.e., NADH) as the current output was further confirmed by comparing the oxidation of glucose (panel A) and NADH (panel B) at the MWCNT-modified electrode (without modification of NAD^+ or GDH), as displayed in Figure 5. At the MWCNT-modified GC electrode, the oxidation

Table 1. Analytical Properties of Dehydrogenase-Based Glucose Biosensors[a]

biosensors	linear range (mM)	limit of detection (μM)	ref
GDH/NAD^+/MWCNT/GC	0.01−0.30	4.81	this work
SPCE/MB	0.075−30	75	1e
GDH-GDI-CHIT/CNT/GC	0.005−0.3	3	2g
PNb-SWNTs/GDH/GC	0.1−8.5	5	1f
Os polymer/CNT paste	up to 0.8	10	1g
BPT/graphite	0.001−2	1	2h
AuNP/thionine/CNT/ITO	0.01−2.56 (dark)	5 (dark)	2i
	0.001−3.25 (light)	0.7 (light)	
Nafion/GDH/NAD^+/CHIT/CNT/GC	0.02−2		7a

[a] SPCE, screen-printed carbon electrode; MB, Meldola's blue; CHIT, chitosan; GDI, glutaric dialdehyde; PNb, poly(nile blue A); SWNTs, single-walled carbon nanotubes; CNT, carbon nanotubes; BPT, bis(benzophenoxazinyl)terephthaloyl chloride; ITO, indium-doped tin oxide; AuNP, gold nanoparticles.

of glucose and NADH take place at +0.90 and +0.25 V, respectively. The potential for the oxidation of glucose at the MWCNT-modified electrode was much higher than that at the GDH/NAD^+/MWCNT-modified electrodes, while the potential for NADH oxidation at the MWCNT-modified electrode was the same as that for the oxidation of glucose at the GDH/NAD^+/MWCNT-modified electrodes. This comparison eventually reveals that glucose was efficiently oxidized under the bioelectrocatalysis of GDH with surface-immobilized, rather than solution-dissolved, NAD^+ as the cofactor. It should be noted that, consistent with the previous report,[10f] the potential for NADH oxidation at the MWCNT-modified electrode was more negative than that at the bare glassy carbon substrate (data not shown), suggesting that MWCNTs employed here not only serve as the electronic transducer and the support to confine NAD^+ cofactor onto electrode surface, but also act as the electrocatalyst for NADH in the dehydrogenase-based electrochemical biosensors.

Figure 6 shows the amperometric response of the GDH/NAD^+/MWCNT-based biosensor toward successive addition of glucose in 0.10 M phosphate buffer (pH 7.0) containing no NAD^+. The electrode was very responsive to glucose, and the current response was linear with the concentration of glucose within a range from 10 to 300 μM (I/nA = 11.7 + 0.474C/μM) with a linear coefficient (γ) of 0.997. For calculation of the limit of detection (LOD), the blank measurements were performed with the biosensor for 17 times in the pure phosphate buffer containing no glucose. The current responses for the blank measurements were 6.92, 7.92, 7.98, 7.20, 6.56, 6.36, 6.12, 5.15, 7.52, 6.80, 7.22, 6.12, 6.15, 7.16, 6.61, 7.80, and 6.20 nA and the standard deviation of the blank measurements (S_b) was calculated to be 0.76 nA. The limit of determination was then calculated to be 4.81 μM with the equation $3S_b/k$, where k is the sensitivity of the biosensor (i.e., 0.474 nA μM^{-1}). The limit of detection and the linear range of the biosensor developed in this study were quite comparable to those of the biosensors reported in the literature, as shown in Table 1, suggesting that the utilization of the strong $\pi-\pi$ stacking interaction between the adenine subunit in the NAD^+ molecule and MWCNTs offers a facile and effective approach to confining the NAD^+ cofactor onto the electrode surface and thereby to general development of integrated dehydrogenase-based electrochemical biosensors.

Conclusions

By taking advantage of the strong $\pi-\pi$ stacking interaction between the adenine moiety in the NAD^+ molecule and MWCNTs, we have demonstrated a simple and effective approach to confinement of NAD^+ cofactor onto electronic transducers for relatively general development of integrated dehydrogenase-based electrochemical biosensors. MWCNTs employed in this study not only serve as the electronic transducer and support to confine NAD^+ cofactor onto the electrode surface but also act as the electrocatalyst for NADH in the integrated dehydrogenase-based electrochemical biosensors. The adsorption of NAD^+ cofactor onto MWCNTs is stable, and the as-prepared GDH-based biosensor with surface-confined NAD^+ cofactor is very responsive toward glucose. Compared with the existing methods for surface confinement of NAD^+ cofactor and thereby for development of integrated electrochemical biosensors, the method demonstrated here is simple and reliable and is thus envisaged to be useful for general development of integrated dehydrogenase-based bioelectrochemical devices, such as biosensors and biofuel cells.

Acknowledgment. The first two authors contributed equally. We gratefully acknowledge the financial support from NSF of China (Grant Nos. 20625515, 20721140650, and 90813032 for L.M. and 20705034 for L.S.), National Basic Research Program of China (973 program, 2007CB935603 and 2010CB33502), and Chinese Academy of Sciences (KJCX2-YW-W25 and KJCX2-YW-H11).